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ETIOLOGY AND RAPID DIAGNOSIS OF HUMAN VIRAL GASTROENTERITIS

Annual Report

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The purpose of this project is to assess the medical and epidemiological importance of various etiologic agents of human viral gastroenteritis through the development of convenient and rapid laboratory methods for their diagnosis. In addition, we are studying the immunological relationships of gastroenteritis viruses to aid in their classification and diagnosis. Particular attention is being paid to the development and utilization of immunoassays to detect etiologic agents, with the preparation and use of monoclonal antibody reagents where possible. During the contract year for this report, we have prepared and characterized monoclonal antibodies that are reactive with a group antigen shared by all known astroviruses, which are small enteric agents that have been associated with some cases of gastroenteritis. Furthermore we have cultivated in cell culture, visualized, and characterized the Marin County agent of gastroenteritis for the first time, and have shown it to be an astrovirus which also reacts with our astrovirus group specific monoclonal antibodies. Current efforts are underway to develop a sensitive monoclonal antibody based im-

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munoassay for detection of astroviruses in stool samples in order to permit for the first time widespread epidemiological studies on the role of these viruses in gastroenteritis. Studies are ongoing with other small gastroenteritis viruses, namely Norwalk virus and calicivirus, in an attempt to cultivate them in cell cultures with the aid ofintestinal secretions provided to us from gnotobiotic swine; in addition efforts are ongoing to prepare monoclonal antibodies to Norwalk virus and human calicivirus. We have also utilized out previously developed monoclonal antibody based immunoassay for the detection of enteric adenovirus types 40 and 41 in human stools to assess the role of these agents in pediatric gastroenteritis in a tropical climate, Thailand. Heretofore, adenoviruses 40 and 41 have been shown to be an important cause of pediatric diarrhea in temperate climates. Our studies indicate that 2 percent of diarrheic children experienced enteric adenoviral diarrhea out of 1,114 ill individuals who were tested, an incidence lower than that reported for temperate climates. During our enteric adenovirus studies, we have also been able to demonstrate that the reported poor growth characteristics of these agents in common cell lines, such as HEP-2, cannot be reliably used for a presumptive diagnosis of adenovirus infection due to enteric adenoviruses 40 and 41. This observation underscores the necessity to use a definitive, adenovirus type and not group-specific assay, such as a monoclonal antibody based immunoassay to diagnose adenovirus 40 and 41 infections. Collaborative epidemiological studies are being carried out with the United States military in Thailand, Peru, and Egypt on the role of gastroenteritis viruses in various overseas populations, in which we have already shown Norwalk virus,enteric adenovirus and rotavirus to cause diarrhea.

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SUMMARY

The purpose of this project is to assess the medical and epidemiological importance of various etiologic agents of human viral gastroenteritis through the development of convenient and rapid laboratory methods for their diagnosis. In addition, we are studying the immunological relationships of gastroenteritis viruses to aid in their classification and diagnosis. Particular attention is being paid to the development and utilization of immunoassays to detect etiologic agents, with the preparation and use of monoclonal antibody reagents where possible. During the contract year for this report, we have prepared and characterized monoclonal antibodies that are reactive with a group antigen shared by all known astroviruses, which are small enteric agents that have been associated with some cases of gastroenteritis. Furthermore we have cultivated in cell culture, visualized, and characterized the Marin County agent of gastroenteritis for the first time, and have shown it to be an astrovirus which also reacts with our astrovirus group specific monoclonal antibodies. Current efforts are underway to develop a sensitive monoclonal antibody based immunoassay for detection of astroviruses in stool samples in order to permit for the first time widespread epidemiological studies on the role of these viruses in gastroenteritis. Studies are ongoing with other small gastroenteritis viruses, namely Norwalk virus and calicivirus, in an attempt to cultivate them in cell cultures with the aid of intestinal secretions provided to us from gnotobiotic swine; in addition efforts are ongoing to prepare monoclonal antibodies to Norwalk virus and human calicivirus. We have also utilized our previously developed monoclonal antibody based immunoassay for the detection of enteric adenovirus types 40 and 41 in human stools to assess the role of these agents in pediatric gastroenteritis in a tropical climate, Thailand. Heretofore, adenoviruses 40 and 41 have been shown to be an important cause of pediatric diarrhea in temperate climates. Our studies indicate that 2 percent of diarrheic children experienced enteric adenoviral diarrhea out of 1,114 ill individuals who were tested, an incidence lower than that reported for temperate climates. During our enteric adenovirus studies, we have also been able to demonstrate that the reported poor growth characteristics of these agents in common cell lines, such as HEP-2, cannot be reliably used for a presumptive diagnosis of adenovirus infection due to enteric adenoviruses 40 and 41. This observation underscores the necessity to use a definitive, adenovirus type-and not group-specific assay, such as a monoclonal antibody based immunoassay to diagnose enteric adenovirus 40 and 41 infections. Collaborative epidemiological studies are being carried out with the United States military in Thailand, Peru, and Egypt on the roles of gastroenteritis viruses in various overseas populations, in which we have already shown Norwalk virus, enteric adenovirus and rotavirus to cause diarrhea.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

CONTENTS

BACKGROUND INFORMATION ON VIRAL GASTROENTERITIS

Acute viral gastroenteritis is an extremely common illness that affects all age groups and occurs in both epidemic and endemic forms (1). It is second in frequency only to the common cold among illnesses affecting United States families under epidemiological surveillance. It is also responsible for some of the common travelers' diarrhea encountered in Latin America, Africa, and Asia. The illness varies in its clinical presentation, but in general it begins with an explosive onset, and consists of varying disabling combinations of diarrhea, nausea, vomiting, low grade fever, abdominal cramps, headache, anorexia, myalgia, and malaise. It can be severe, indeed fatal, in the elderly, infant, debilitated, or malnourished patient.

Viral gastroenteritis occurs primarily in two epidemiologically distinct clinical forms (1). One entity is characteristically epidemic and is responsible for family and community-wide outbreaks of gastroenteritis among older children and adults. In recent years, one agent, Norwalk virus, has been shown to be responsible for about 40 percent of these disease outbreaks in the United States. Other Norwalk-like viruses have also been discovered such as Hawaii agent and Snow Mountain agent, and although they have not been well studied epidemiologically, they are likely to be responsible for many more epidemic cases of this illness.

The second clinical entity is usually sporadic, occasionally epidemic, and occurs predominantly in infants and young children (1). However, as noted below it can occur in adults. This form of illness typically produces severe diarrhea that commonly lasts for five to eight days and is usually accompanied by fever and vomiting. Rotavirus, which was discovered during the 1970's, is responsible for nearly one half of the cases of this clinical entity requiring hospitalization. Although the major target of rotavirus is the very young, it can produce surprisingly severe clinical disease in adults (1,2).

Breakthroughs in determining the medical importance of Norwalk virus and rotavirus occurred primarily because of the development of immunoassay techniques to recognize these viruses in stool samples and to measure antibodies to them in infected individuals. For Norwalk virus, these assays are currently available in only a few research laboratories (3,4) including that of the principal investigator. This is because the procedure requires use of precious limited human volunteer materials (stools and sera). The assay has more recently been made more efficient in detecting Norwalk virus antigen in stools through the use of an enzyme-linked immunoassay (EIA) instead of a radioimmunoassay (RIA)(5). Together with our collaborators, our use of these immunoassays has shown a major role for this virus in producing in the U.S. clam and oyster associated gastroenteritis, as well as some cases of travelers' diarrhea in Mexico and Thailand (6-8).

As for rotavirus, use of immunoassay techniques to detect the virus is now common and is employed routinely in many clinical diagnostic laboratories (9). More recently, a monoclonal antibody based EIA that we developed for detection of rotavirus (10) has been shown to be more sensitive and specific than polyclonal antibody tests and has eliminated specificity problems with stool samples from young infants. We have used rotavirus immunoassays to establish the role of rotavirus in several nations around the world, including

travelers' diarrhea experienced by U.S. military populations overseas (7,11-15).

The roles of other enteric viruses in gastroenteritis are poorly understood, and because of the medical importance of infectious diarrhea, there is clearly a major need to establish the significance of different viruses that may be involved. Comparative studies on their occurrence, however, have been infrequent and usually limited to electron microscopy (15,17). The major obstacle in evaluating the relative importance of the non-rotavirus and non-Norwalk virus enteric viruses as causative agents of gastroenteritis has been the lack of convenient methods for their diagnosis. In addition, for appropriate treatment and control measures to be initiated, rapid as well as convenient methods are required, but are also unavailable for most of these gastroenteritis viruses. Further, many of these viruses are difficult to cultivate or have not been cultivated in cell culture, which has inhibited characterization studies.

Among these agents, the evidence currently seems strongest that "enteric" adenoviruses are medically important pathogens like rotavirus and Norwalk virus. These adenoviruses differ from the well characterized conventional serotypes of adenoviruses which are propagated in standard tissue cultures and are not commonly associated with gastroenteritis. The "enteric" adenoviruses are recognized by electron microscopy in stools and cultivatable inefficiently in an adenovirus transformed cell line, Graham 293 (18). Two "enteric" serotypes (types 40 and 41) have been identified and in a limited number of studies performed to date, have been highly associated with gastroenteritis in infants and young children and much less commonly found in asymptomatic children (1,19). The potential role of enteric adenoviruses in travelers' diarrhea or in disease in adults has not been studied. Convenient and specific immunoassays to detect enteric adenoviruses have been greatly needed, and would permit an understanding of their epidemiology as has already occurred with the use of immunoassays to study rotavirus and Norwalk virus. During the 1986-1987 contract year, we prepared monoclonal antibodies specific for adenovirus types 40 and 41. These antibodies were characterized and used in an EIA format to detect the enteric adenoviruses in known positive diarrheal stool specimens with 95 to 98 per cent sensitivity and specificity (20,21). We are now using this enteric adenovirus monoclonal antibody EIA to assess the epidemiology of this infection in several populations.

Caliciviruses have also been associated with diarrheal disease in humans (1,22). These agents are currently detected solely by electron microscopy and more convenient assays for their detection are needed so that their epidemiology can be studied. Norwalk virus possesses a single structural protein, characteristic of a calicivirus (23), and the two agents are of similar size and general shape (albeit, differing somewhat in virion surface structure). Thus, the possibility of relatedness between these two enteric viruses exists and was studied by us during the 1986-1987 contract year. We demonstrated that antigenic characteristics are shared between calicivirus and Norwalk virus based on our detection of seroconversions to Norwalk virus in patients experiencing gastroenteritis due to a strain of calicivirus (24,25). These two agents, therefore, may belong to the same family of viruses, as also may Snow Mountain agent for which we have also found seroconversions to Norwalk virus in some affected patients (26). These

serological cross-reactions demonstrate the need for viral antigen specific detection methods for calicivirus such as we previously developed for Norwalk virus.

Astroviruses, like caliciviruses, are small (27-35nm in diameter) and have been identified by electron microscopy in the stools of some patients with gastroenteritis (1,27). Astroviruses have been reported to be cultivatable in cell culture (28,29). However, simple diagnostic procedures have not been developed. Thus, the extent of the role of astroviruses in human diarrheal disease is not known. During the 1986-1987 contract year, we were able to confirm the reported in vitro cultivation of 4 of 5 serotypes of human astrovirus, and thereby to purify sufficient viral antigen to prepare monoclonal antibodies reactive against a common antigen shared by multiple astrovirus serotypes (30). This offers the practical possibility for developing immunoassays to assess the medical importance of astroviruses in human viral gastroenteritis.

ANTIGENIC CHARACTERIZATION OF CELL CULTIVATED ASTROVIRUS SEROTYPES AND DEVELOPMENT OF ASTROVIRUS-SPECIFIC MONOCLONAL ANTIBODIES

During the current (1987-1988) contract year, we have extended our studies on the characterization and detection of cell cultivated human astroviruses, for which there are 5 serotypes as defined by Kurtz (29). By use of type-specific rabbit antisera, serotypes of astrovirus were readily distinguished by immunofluorescence (IF) of virus infected cells, as shown in the accompanying Table 1.

Table 1. Antigenic relationships of astrovirus serotypes by immunofluorescence

Astrovirus serotype*	Reciprocal titer of antibody to serotypes					
	1	2	3	4	5	Monoclonal
1	1,600	<25	<25	<25	<25	100,000
2	<25	12,800	<25	100	<25	10,000
3	80	<20	320	<20	<20	20,000
4	<25	<25	<25	3200	<25	100,000
5	50	100	<25	<25	800	100,000

*Serological data for serotype 3 are from a previous report (29) as we were not able to cultivate serotype 3 for these comparisons. The monoclonal antibody reactivity for serotype 3 was determined by Dr. Kurtz in Oxford, U.K.

In contrast, however, use of type-specific rabbit antisera in an EIA format revealed a high degree of cross-reactivity among astrovirus serotypes, indicating presence of a group antigen. The EIA findings are shown in Table 2.

Table 2. Antigenic relationships of astrovirus serotypes by EIA

Astrovirus serotype*	Reciprocal titer of antibody to serotypes					
	1	2	3	4	5	Monoclonal
1	6,400	12,800	<100	<100	400	<100
2	6,400	6,400	<100	800	400	1,000,000
3	NT	NT	NT	NT	NT	10,000
4	400	<100	<100	1600	<100	1,000,000
5	6,400	800	400	400	1,600	1,000,000

*Astrovirus type 3 virus used was seed virus as we were not able to cultivate this one serotype. NT= not tested.

The difference in the degree of serotypic cross-reactivity between the IF and EIA results may indicate the manner in which viruses adsorb to solid-phase surfaces. This type of adsorption may result in degradation of the virion and release of group antigenic determinants, as has been reported for coxsackie viruses (31,32).

We also prepared monoclonal antibodies against purified astrovirus type 2 antigen and selected them on the basis of group antigen reactivity. These antibodies were reactive by IF and/or EIA (see Tables 1 and 2) with the 4 astrovirus serotypes that we could cultivate as well as with the previously reported cell cultivated astrovirus type 3. These findings also indicate that the astroviruses share a group antigen. The lack of reactivity of type 1 astrovirus in the monoclonal antibody EIA (Table 2) is currently unexplained, as this serotype was readily detected in infected cell cultures by IF with the monoclonal antibody, and could also be detected in 2 stools by monoclonal antibody EIA.

These data have been accepted for publication in the Journal of Infectious Diseases (30). The monoclonal antibodies, reacting broadly with human astroviruses, hold promise for the rapid diagnosis of astrovirus infection and for determining the importance of these viruses as agents of viral gastroenteritis. Currently, we are attempting to prepare monoclonal antibodies to astrovirus types 1 and 5 (the latter type being related to the Marin County agent as indicated below) in order to better characterize these viruses.

PROPAGATION AND IMMUNOLOGICAL CHARACTERIZATION OF MARIN COUNTY STRAIN OF ASTROVIRUS, AND ITS DETECTION BY MONOCLONAL ANTIBODY

Marin County Agent (MCA) was first described in 1981 as the result of an investigation of an outbreak of gastroenteritis among patients and staff in a convalescent home (33). Examination of fecal samples from patients and

from an adult volunteer by electron microscopy (EM) revealed the presence of large numbers of small round viruses 27 nm in diameter. MCA had been placed within the Norwalk virus group although immune EM (IEM) and radioimmunoassay (RIA) tests indicated that it was antigenically distinct from Norwalk virus and Hawaii virus (33).

We obtained the MCA present in a human stool specimen, through the courtesy of Dr. H. Greenberg (Stanford), and initiated studies to try to cultivate and characterize the agent. We were successful in being able to isolate and serially propagate (18 passages to date) the MCA in human embryonic kidney cells by use of trypsin-containing medium in the cell cultures. We used the same methodology that had proven successful for the cultivation of human astroviruses (28,30) in cell cultures. We further showed that the morphology by electron microscopy of MCA in stool is characteristic of astroviruses and showed reactivity by immune electron microscopy (IEM) with astrovirus type 5 antiserum. Acute and convalescent sera from an MCA-infected patient showed seroconversion to astrovirus type 5 by EIA and IEM, and also to astrovirus type 1 by IEM. The IEM data are shown in Table 3.

Table 3. Serological Reactivity of Marin County Agent by IEM

Serum	Reciprocal IEM antibody titer to virus types			
	MCA*	MCA+	Astrovirus type 1*	Astrovirus type 5+
MCA acute ++	<50	<100	50	<50
MCA convalescent	1600	800	800	≥1600
Astrovirus type 1 **	<50	<50	640	<50
type 2	<50	<50	<50	<50
type 3	<50	<50	<50	<50
type 4	<50	<50	<50	<50
type 5	200	100	<50	6400

*Stool - derived, + Cell culture propagated, ++Human, **Rabbit

In additional studies, we have shown that immunofluorescence of MCA propagated in cell culture showed positive reactivity with both astrovirus types 1 and 5 antisera and with our group-reactive astrovirus monoclonal antibody. Therefore, we can now conclude that MCA can be propagated by techniques used for other astroviruses and is serotypically an astrovirus type 5 that cross-reacts in some tests with astrovirus type 1.

A preliminary report of our data has been published in The Lancet (34).

These findings now characterize one agent, originally felt to be a Norwalk-like virus, as an astrovirus, using in vitro cultivation, IEM and monoclonal antibody techniques. They lend conceptual support to studies attempting to define other candidate small viral enteric agents suspected of causing

gastroenteritis.

We have not applied MCA antigen detection techniques to random testing of stools, but we were able to readily detect antigen by EIA in the one stool sample we had which was known to contain the virus. This suggests that an MCA EIA could be developed for screening stool samples in outbreaks of gastroenteritis. The ability to isolate MCA in cell culture directly from stool, and its recognition as an astrovirus will facilitate studies on the epidemiology of MCA and its significance within the astrovirus group.

STUDIES TO DEVELOP IMMUNOASSAY FOR DETECTION OF ASTROVIRUSES IN STOOL SAMPLES

Our studies with the immunological characterization and in vitro cultivation of human astroviruses, including the Marin County agent, now permit us to attempt to develop an immunoassay for the detection of these viruses in human stool samples. (As noted above, we have already detected these viruses by EIA in infected cell cultures). Key reagents to these efforts should be astrovirus group reactive monoclonal antibodies, such as we have already prepared. We have been kindly provided 30 astrovirus electron microscopy positive stool samples by Dr. David Cubitt (London) to aid in this effort. To date, we have examined in a preliminary study 12 of these samples, using an EIA in which polyclonal rabbit antiserum is used as a coating reagent and monoclonal antibody as the detector reagent. When looked at in this pilot study, 4 of the 12 astrovirus-positive stool samples reacted in the EIA. Future efforts will be carried out to optimize the sensitivity of this EIA. We are, for example, evaluating different combinations of polyclonal antibodies for the coating procedure, varying diluents in which to suspend test antigens, and we may also evaluate monoclonal antibodies as both coating and detector reagents. Our efforts to develop an EIA to detect astrovirus in stool samples, if successful, will then permit us to assess the medical importance of astrovirus gastroenteritis.

IN VITRO CULTIVATION STUDIES WITH CALICIVIRUS AND NORWALK VIRUS

Encouraged by our success with the in vitro cultivation of human astroviruses, we have also been undertaking efforts to grow human caliciviruses and Norwalk virus in cell culture. Stocks of human stool specimens containing caliciviruses, as well as corresponding paired acute and convalescent sera, have been provided to us by D. Cubitt (London). These fecal samples are defined electron microscopically and clinically as being derived from naturally occurring calicivirus disease outbreaks. Dr. Cubitt is a primary collaborator with us on our calicivirus studies and is a major authority on these agents. During the current contract year, he spent three months in our laboratory, helping us to expand our capabilities in the diagnosis of caliciviruses and other small enteric viruses by electron microscopy. Our Norwalk virus particle-containing fecal and paired serum reagents are derived from our volunteer studies performed a decade ago.

Two main approaches are being carried out, attempting to cultivate human calicivirus and Norwalk virus. The first relies upon an observation by Flynn and Saif (35), who were able to cultivate a previously noncultivatable pig enteric calicivirus in vitro with the use in cell cultures of small intestinal fluids derived from uninfected gnotobiotic piglets. Cultivation

was demonstrated by the presence of fluorescent-stainable viral antigen in inoculated cells. Dr. Saif has kindly provided us with some of these intestinal fluids which we are using in human calicivirus and Norwalk virus inoculated cell cultures. To date, Norwalk inoculated cultures have been passaged 7 times without evidence of virus-specific IF-stainable antigen. However, Saif's studies required even further passage levels to establish predictable replication. We have not yet started our human calicivirus studies using the small intestinal fluids.

The second approach to cultivation of Norwalk and calicivirus is the use of trypsin containing culture media with centrifugation of inoculated cells onto coverslips for IF studies. This is the technique successfully used by Kurtz (28) and us (30) for cultivation of astrovirus. To date, Norwalk virus inoculated cultures are negative to the sixth passage level and studies with calicivirus have not yet started.

Further attempts to detect Norwalk virus and calicivirus are being made by means of producing monoclonal antibodies to these agents. This is a complicated technical problem because the immunogen for these studies contains human stool materials in addition to virus. Our prior attempts to produce monoclonal antibodies to Norwalk virus have failed, probably due to interference in the assay to detect virus-specific monoclonal antibody by antibodies also made to human immunoglobulins in stool. We are approaching this problem by trying to deplete immunoglobulins from the stool containing Norwalk virus inoculum through use of acid treatment to dissociate virus antigen-antibody complexes. Resultant released virus is being purified by differential centrifugation for use as the immunogen to prepare monoclonal antibodies.

It is our assumption that it is logical to perform the same cultivation and monoclonal antibody studies with human caliciviruses as with Norwalk virus due to the evidence that these two agents are related in terms of their protein composition (23) and immunological characteristics (24,25). In this regard, our work on the detection of seroconversions to Norwalk virus in patients experiencing gastroenteritis due to a strain of calicivirus was published during the current contract year in the Journal of Infectious Diseases (24).

DEFINITIVE DETECTION OF NORWALK VIRUS BY ANTIGEN TESTING IN AN OUTBREAK SETTING

In collaboration with the Erie County Health Department and SUNY, Buffalo, we have recently linked Norwalk virus in a definitive fashion with two foodborne outbreaks of gastroenteritis. What is particularly of interest here is not the epidemiology (which has been well defined previously) but the confirmation in an outbreak setting of the utility of Norwalk virus antigen testing by EIA in stool samples, the technique for which we have previously reported (5). Seven of 21 stool samples (33%) from ill persons were positive for Norwalk virus antigen in these outbreaks (in which 24 of 34 paired sera seroconverted to Norwalk virus antigen). All seven of the patients with Norwalk virus-positive stools were also positive by seroconversion. The detection of Norwalk virus antigen, rather than merely a Norwalk antibody seroconversion, is important because it provides a definitive virus association unlike seroconversion. This is because, as we have recently shown (24,26), pa-

tients seroconverting to Norwalk virus can also seroconvert to human caliciviruses and Snow Mountain agent as these agents are immunologically related. Our data with the SUNY, Buffalo group on Norwalk virus antigen detection have recently been accepted for publication by the American Journal of Epidemiology (36).

INCIDENCE OF ENTERIC ADENOVIRUSES AMONG CHILDREN IN THAILAND AND SIGNIFICANCE OF THESE VIRUSES IN GASTROENTERITIS

As outlined above in the section "Background Information on Viral Gastroenteritis," we have previously developed a monoclonal antibody based EIA for the direct detection of the enteric adenoviruses (types 40 and 41) in stool specimens (20,21). We have now been able to use this assay to assess the incidence of these viruses as a cause of pediatric gastroenteritis in a tropical climate, Thailand. These studies have been carried out in collaboration with Drs. Peter Echeverria and David Taylor of the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. Heretofore, the only reports of the incidence of adenoviruses types 40 and 41 in pediatric diarrheal disease have come from countries with temperate climates, and have revealed their presence in about 6 to 8 per cent of cases (37).

In our collaborative study, stools were collected by the AFRIMS group from children under age seven over a one year period at an outpatient clinic in Bangkok, Thailand. Stools were tested from 1,114 children with gastroenteritis and from 947 children without gastroenteritis. Each stool was tested for adenovirus (Ad) group antigen and for specific enteric Ad types (Ad40 and Ad41) by our monoclonal antibody enzyme immunoassays. We found that 4.4% (49/1,114) of children with gastroenteritis and 1.8% (17/947) of children without gastroenteritis were positive for Ad group antigen. In tests for specific enteric Ad types, 2.0% (22/1,114) were positive in children with gastroenteritis and 0.6% (6/947) in children without gastroenteritis. There was a significant correlation of gastroenteritis with non-enteric Ad types ($p<0.02$) as well as with the specific enteric Ad types ($p<0.01$). By comparison, 19.7% of children with gastroenteritis and 0.7% in those without gastroenteritis were positive for rotavirus infection. These studies are summarized in Table 4.

Table 4. Incidence of enteric adenoviruses, non-enteric adenoviruses, and group A rotavirus in stools from Thai children with and without gastroenteritis.

		No. (%) virus positive		
		Adenoviruses		
Patient group	No. tested	Enteric	Non-enteric	Rotavirus
Children with gastroenteritis	1,114	22 (2.0)[a]	27 (2.4)[b]	220 (19.7)
Children without gastroenteritis	947	6 (0.61)[a]	11 (1.2)[b]	7 (0.7)

[a] $p< 0.01$ [b] $p<0.02$

There were no significant differences in the association of bacterial or parasitic infections with enteric Ad or non-enteric Ad infections in either group of children studied.

The pattern of infection with enteric Ad and non-enteric Ad, their association with gastroenteritis, and co-infection with other pathogens is similar to that found in a study of 416 ill children in a temperate climate, Uppsala, Sweden (38). The percentage of total Ad specimens identified as enteric was somewhat higher in that study, 59% (33/56) compared to the 45% (22/49) that we found, and the overall incidence of enteric Ad as well as total Ad was higher in that population than in the one we studied (7.9% and 13.5% versus 2.0% and 4.4%, respectively).

We can conclude that the enteric adenovirus types 40 and 41 are a cause of gastroenteritis in the Thai children studied. However, the incidence of enteric adenovirus in this group is less than that found in most reports to date from areas with temperate climates. Our data are being prepared for publication.

ISOLATION AND PROPAGATION OF ENTERIC ADENOVIRUSES IN HEP-2 CELLS

Adenoviruses have been isolated from stools for over 20 years, but it is only in recent years that the specific adenovirus types 40 and 41 have been closely associated with gastroenteritis. These enteric types were originally described as viruses which could be visualized in stools of patients with gastroenteritis by electron microscopy (EM), but could not be cultivated in cell cultures generally used in diagnostic virology laboratories for isolation of respiratory adenoviruses. Subsequently, it was found that the enteric types could be cultivated in Graham 293 cells, an adenovirus type 5-transformed human embryonic kidney (HEK) cell line (18).

Based on these observations, it has become axiomatic that detection of adenoviruses in stools by EM, and failure to isolate them in cell lines known to support growth of other adenovirus types, is presumptive evidence for the enteric types. Conversely, it is now assumed in epidemiological studies on adenoviruses that virus replication in HEp-2 or other conventional cell lines can be used to designate adenovirus isolates as non-enteric (39).

During the course of our studies undertaken to evaluate our monoclonal antibody based EIA for enteric adenovirus diagnosis (21), it appeared that some of the enteric types could be isolated in HEp-2 cells as well as in Graham 293 cells. We therefore set out to examine in detail the relative efficiency of primary isolation of enteric adenoviruses 40 and 41 in these two cell lines.

Eighty-two stool samples from children with gastroenteritis in Canada, England, and Thailand, which had been shown to contain adenovirus (Ad) antigen by a group-specific EIA and/or Ad particles by electron microscopy, were tested for primary isolation of enteric Ad in HEp-2 and Graham 293 cells. It was found that there were 73 of the 82 Ad isolates which could be typed as Ad 40 or Ad 41 by our type-specific monoclonal antibody EIA and by analysis of Sma I endonuclease digests. Of these 73, 30 (41%) could be isolated in HEp-2 cells, which included 43% (9/21) of those typed as Ad 40 and 40% (21/52) of those typed as Ad 41. Based on these results, the growth

characteristics of Ad in Hep-2 cell cultures, commonly used to distinguish enteric from non-enteric Ad types, are not valid for either diagnosis or epidemiological studies. For the samples we studied, use of these non-definitive criteria would result in underestimating the incidence of enteric Ad in viral gastroenteritis. This study reemphasizes the importance that a definitive, type specific test such as a monoclonal antibody based EIA, needs to be used to diagnose enteric Ad infections and to determine the role of enteric Ad in epidemiological studies of viral gastroenteritis. These data are being prepared for publication.

COLLABORATIVE EPIDEMIOLOGICAL STUDIES WITH THE MILITARY

Several collaborative studies on the role of viral agents in gastroenteritis have been performed with scientists with the U.S. military during the current contract year.

One study is that performed with Drs. Peter Echeverria and David Taylor of AFRIMS on the incidence of enteric adenoviruses types 40 and 41 in pediatric diarrhea in Bangkok, Thailand. This study is described in detail above in the section entitled "Incidence of enteric adenoviruses among children in Thailand and significance of these viruses in gastroenteritis."

In other studies with the AFRIMS group, stools collected by the AFRIMS group from 414 Thai children with diarrhea less than 5 years of age were evaluated for presence of rotavirus by monoclonal antibody EIA. Fifty-five (13.3%) were positive. These samples are currently being tested for adenovirus 40 and 41 by monoclonal antibody EIA. Dr. Echeverria is also prospectively studying over 1 year, the incidence and etiology of diarrhea in sick infants under 6 months of age and their age matched controls in Bangkok. We plan to study samples from this study collected by the AFRIMS group for the presence of enteric adenoviruses.

We have performed a second collaborative study with the Naval Medical Research Institute Detachment, Lima, Peru (Cdr. F.S. Wignall), and Naval Medical Research Institute, Bethesda (Dr. A.L. Bourgeois) on the Norwalk viral etiology of travelers' diarrhea in U.S. military personnel visiting seaport cities of Central America, South America and West Africa. In the first collaborative study (outlined in last year's Annual Report), 12 per cent of 137 cases of diarrhea were associated with Norwalk virus based on seroconversions to the virus. In this year's study (Blue Horizon and UNITAS studies), seroconversions to Norwalk virus were noted in 12 of 99 representative servicemen studied by the Navy group, again indicating a role for this pathogen in diarrhea among servicemen visiting seaport cities. The 12 per cent seroconversion rates were strikingly similar between the 2 years of study.

Another study is underway with the U.S. Medical Research Unit #3 in Cairo,Egypt, with Dr. R. Haberberger. This study is evaluating 100 diarrheal stools, collected by Dr. Haberberger and associates, for Norwalk virus and enteric adenoviruses. These specimens were collected from Operation Brightstar, during a 10 to 31 day period (July-August 1987) in which 4600 U.S. troops were on land based tours in Egypt. One hundred eighty-three soldiers developed diarrhea, of which half have yielded no bacterial or parasitic pathogens.

Finally, the scientific project officer, Dr. L. Binn, has suggested our collaboration in the study of enteric non-A, non-B hepatitis virus. We have offered to provide WRAIR aliquots of clinical samples of serums and stools collected over the years in our studies on gastroenteritis and are also prepared to test specimens of the enteric non-A, non-B hepatitis virus for its relatedness to morphologically similar gastroenteritis viruses. During the current contract year, we have also shipped astrovirus and antibody to astrovirus to Dr. Binn, WRAIR.

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PUBLICATIONS OF WORK SUPPORTED BY THIS CONTRACT

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